Separation and Estimation of Organic Acids on Paper Chromatograms

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In the present paper a method is described for the qualitative separation and quantitative estimation of the ammonium salts of a wide variety of acids on paper chromatograms using single-phase mixtures of *n*-propanol and concentrated aqueous ammonia as the developing solvents. The method for the qualitative separation of organic acids was briefly mentioned in a previous paper describing the separation of phosphoric esters on paper chromatograms (Hanes & Isherwood, 1949), and since then a number of workers (Brown & Hall, 1950; Brown, 1950; Hiscox & Berridge, 1950; Kennedy & Barker, 1951; Brown, 1951; Long, Quayle & Stedmann, 1951) have employed basic solvent mixtures to effect a qualitative separation of organic acids.

The method developed for the quantitative determination of the ammonium salts of organic acids is based upon a simple colorimetric procedure, and is accurate to $\pm 10\%$ when about 50 μg. of organic acid is being determined. The method rests upon the fact that under suitable conditions the colour of a selected indicator is unaffected by the presence of ammonia, so that the ammonium salts of organic (and other) acids behave as free acids, the colour of the indicator being diminished in proportion to their concentrations. The principle has been applied to determining both volatile and non-volatile organic acids on paper chromatograms.

The same principle could obviously be applied to the determination of organic bases which could be separated as salts of a weak acid (e.g. by separating chromatographically in an appropriate solvent containing acetic acid). The acetates of the bases could then be determined by their effect upon the colour of an indicator (of pK 2–3) under conditions in which it would be unaffected by the presence of acetic acid in small amount.

For most of the experiments described in the present paper artificial mixtures of organic acids were used, but satisfactory results were obtained on extracts of biological materials by a modified procedure in which the acids were first separated from the bulk of the other solutes by a method using a silica gel column (Isherwood, 1946) before being examined on the paper chromatogram.

Since this work was completed, Reid & Lederer (1952) have published a method for the estimation of the lower fatty acids (C_2–C_7) based on the area of the spot on a chromatogram.

EXPERIMENTAL

Reagents

The various organic acids used in this study were examined on a paper chromatogram using *n*-propanol: concentrated aqueous NH_3 as solvent and only those which appeared to be homogeneous were accepted.

For the quantitative analysis of the acids on paper the propanol used was purified as follows. It was refluxed for 1 hr. with solid NaOH, and then distilled through a fractionating column, rejecting the first fraction (b.p. range, 93–95°) and collecting the next fraction (b.p. range, 96–98°). Commercial propanol, which is adequate for the qualitative separation of the acids, appears to contain traces of an organic acid which seriously affects the blank value of the paper in the method described below. The concentrated NH_3 was A.R. grade.

The ethanol used for preparing the thymol blue-glycine buffer reagent was purified by refluxing for 1–2 hr. over solid NaOH and distilling in an all-glass apparatus. Ordinary absolute ethanol contains sufficient aldehyde and acidic impurities to affect the blank values of the thymol blue reagent.

Analytical procedures

Chromatography. The apparatus and conditions used for the development of the paper chromatograms were essentially the same as those described by Hanes & Isherwood (1949) for phosphoric esters. Solutions of the organic acids, normally as ammonium salts and in concentrations not exceeding 0·3 N, were spotted at 3 cm. intervals along the starting line of a sheet of filter paper by means of a micropipette of volume about 4 μl. This consisted of a thin-walled spindle-shaped capillary tube conveniently mounted at the mouth of a glass tube. It is essential to calibrate the pipette for the particular solution to be used, if the surface tension (due to the presence of organic solvent) or viscosity are appreciably different from those for water.

The paper chromatograms were developed with a single-phase mixture of *n*-propanol and aqueous NH_3. The actual proportions of the components varied with the group of

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acids to be separated; solvents with more water were useful in separating the acids with more than one hydroxyl or carboxyl group. A very convenient mixture for the lower fatty acids was *n*-propanol: conc. aqueous NH_3 (sp.gr. 0·880) (70:30, v/v). For the dicarboxylic acids such as tartaric and malic, the solvent contained *n*-propanol: water: conc. NH_3 (60:20:20, v/v).

Spraying reagents. To detect the various organic acids the developed chromatograms were sprayed (3–5 ml./100 cm.^{2}) with one of the following solutions:

(1) *Thymol blue.* 0·1% (w/v) in water to which sufficient 0·1 N-NaOH had been added to render the solution deep blue, pH 10. When this was sprayed on to a paper in a CO_2-free atmosphere, the paper was coloured light blue. The ammonium salts of the acids showed up as yellow spots on a deep-blue background, cations other than ammonium showing up as spots with a deeper blue colour than the background. For spraying, the papers were suspended in an accumulator jar by clamping one end of the paper between two glass plates which closed the open end. The jar had a hole bored in a central position through one of the vertical sides so that the orifice of a spray could be inserted and the paper sprayed. The developed chromatograms were suspended in the jar for at least 10–20 min. while a stream of CO_2-free air was blown through before being sprayed. The compressed air operating the spray was also free from CO_2. Once the papers were removed from the jar the background colour faded but lasted long enough for the positions of the acids to be outlined. The indicator is very sensitive and will even detect phenols on the paper.

(2) *Ammoniacal silver nitrate.* This was prepared by diluting 0·1 M-$AgNO_3$ with one-fifth its vol. of conc. aqueous NH_3. After spraying, the papers were heated at 105° for periods up to 30 min. The reagent is very sensitive and reacts with a wide variety of substances. Reducing substances such as sugars, aldehydes, hydroxy acids and phenols give dark brown-violet spots, the shade of which is often characteristic of the type of substance. Some non-reducing acids inhibit the colour developed by the paper itself and the position of these acids is revealed by a white spot on a light-brown background. Salts and amino acids also affect the reagent, but usually to a much smaller extent. This reagent is not specific for acids. Chromatograms sprayed with this reagent are usually compared with similar chromatograms sprayed with the thymol blue reagent in order definitely to identify unknown compounds as acids. The papers sprayed with the silver reagent are useful as permanent records.

Purification of filter paper. The filter paper used was purified by chromatographic washing with 2 N acetic acid, distilled water and 10 N-NH_3. Purification of the paper did not affect the general movement of the various acids on the chromatogram but did give distinctly better resolution. The spots showed less tendency to trail, 'ghost' spots were not left behind on the starting line and the sensitivity of methods for the location of acid spots was improved because the paper had been freed of soluble acidic and reducing impurities. For quantitative work the use of washed paper was essential. Whatman no. 1 paper was used for both qualitative and quantitative work, and no. 3 for preparative work.

The apparatus used for the chromatographic washing of filter papers in the present study consisted of a divided Perspex trough which would take a block of about 60 sheets of Whatman no. 1 paper (11·25 in. wide). The design of the trough was such that when the two sides were firmly clamped together the block of papers was held firmly without any gaps or spaces through which liquid could percolate. The arrangement is shown in Fig. 1. For assembly the block of paper was bevelled very slightly with a sharp razor at A and A_1 and then inserted into the slot in the trough. The sides were then clamped together, locating bolts at each end ensuring that the two halves were correctly positioned. The slight gaps which might be present at A and A_1 were closed by ramming a little filter-paper pulp into the crevices. The trough with the block of paper was then suspended in a conveniently sized tank and the trough filled with the washing liquid. The whole apparatus was covered with Alkathene sheeting to keep out dust. The fit of the block of paper in the trough was tested by measuring the flow of liquid through the paper; about 1 l. should percolate every 24 hr. The papers were washed until no more soluble material was removed. This was tested by chromatographically extracting a small piece of the paper (5 × 20 cm.) with 10 ml. of 10 N-NH_3 and evaporating the extract to dryness. Less than 0·3 mg. of residue should remain.

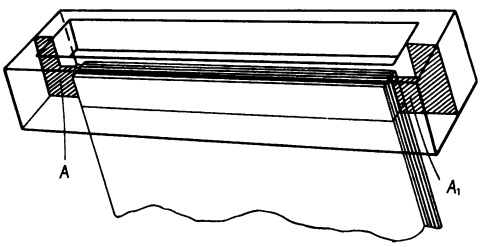

Fig. 1. Apparatus for chromatographic washing of filter paper. For explanation see text.

Quantitative estimation of the ammonium salts of organic acids. The indicator solution was prepared by adding 1 ml. of a 0·05 M solution of the sodium salt of glycine (made by dissolving 3·75 g. glycine in 1 l. of 0·05 N-NaOH, free from carbonate) to 450 ml. of 35% (v/v) aqueous ethanol containing 8 mg. of thymol blue. The solution was stirred by a stream of CO_2-free air. To the solution was added 1·4 ml. of 0·1 N-NaOH, which should bring the intensity of the blue colour to about 80% of the maximum value (i.e. that observed when excess of NaOH is added) as measured under standard conditions in the colorimeter. The solution was stored in the 500 ml. reservoir attached to a 10 ml. burette with an automatic zero point. The bottle and burette were protected against the entry of CO_2.

The procedure in the case of a synthetic mixture or a relatively pure solution of the ammonium salts of the acids was as follows: 4 μl. or an appropriate quantity to give a reading in the colorimeter equivalent to about 40% neutralization of the indicator-buffer solution, was applied to the starting line of a paper chromatogram (Whatman no. 1, washed). The paper was developed with propanol: aqueous NH_3; the proportions depended on the acids to be separated. After development, the paper chromatograms were suspended in accumulator jars and a stream of dry CO_2-free air was blown through for at least 2 hr. The dry papers were then cut into strips corresponding to the chromatograms of the various spots originally applied to the starting line and the individual acids on these located by reference to a guide chromatogram. The piece of paper (usual size 3 × 3 cm.) containing the ammonium salt of the acid was cut out of the chromatogram and then folded twice in one direction and

once in the other, using a clean forceps. Each folded paper was inserted into a clean dry colorimeter tube and 3 ml. of 35% (v/v) aqueous ethanol were added. A slow stream of CO_2-free nitrogen or air was bubbled through the liquid by means of a fine capillary tube inserted into the colorimeter tube. This stream of CO_2-free gas was continued throughout subsequent operations until just before the colour was measured in the colorimeter. In addition, the tube was usually partly closed by a clean rubber bung to prevent the entry of CO_2. After 5 min. the paper was carefully lifted out of the liquid with a clean platinum wire, pressed against the side of the tube with a clean glass rod and then removed. 4 ml. of the thymol blue reagent were added, the tip of the burette being inserted well into the colorimeter tube. After 1 min. the capillary tube was withdrawn and the test tube promptly closed with the bung. The contents were shaken and the colour was measured in the colorimeter, using a green filter (Ilford 626).

The measurement of the blank must be carried out on a piece of paper from the same chromatogram. Even washed paper did not behave the same before using as a chromatogram as afterwards. In a typical example the blank readings were as follows: without any paper, 65; with the addition of paper from a freshly dried (in absence of CO_2) paper chromatogram, 68; with addition of washed paper stored in the laboratory, 63. The discrepancy between the papers is serious in the case of small amounts of acid.

In the case of extracts of biological material, the acids were first separated from the bulk of the other solutes present before estimating by the procedure described above. The procedure using silica-gel columns described by Isherwood (1946) was employed to extract all the acids, volatile and non-volatile. The plant juice acidified with H_2SO_4 was absorbed into silica gel and extracted in the form of a column with 50% (v/v) *n*-butanol: $CHCl_3$. The solvent which issued from the column was then neutralized with a slight excess of 20 N-NH_3. The aqueous phase which separated contained a concentrated solution of the ammonium salts of all the acids and was used without further purification for the procedure described above.

Chromatographic extraction of filter-paper strips. The apparatus used was similar in principle to that ordinarily employed for paper chromatography except that the trough was made of thin Perspex sheet and could be readily removed from the chromatogram jar. The paper strips were suspended from the trough and the lower end of each paper cut to a point. A small stainless steel or glass clip was attached to the point. The liquid percolating through the paper dripped into a cup supported by an adjustable mounting.

RESULTS

Behaviour of carboxylic acids on paper chromatograms

A large number of mono-, di-, and tri-carboxylic acids were examined using different mixtures of *n*-propanol and concentrated aqueous ammonia as solvents. The R_F values are presented in Table 1.

Table 1. *R_F values of organic acids on paper chromatograms using mixtures of* n-*propanol and concentrated ammonia as developing solvents*

(Temp. 20°, Whatman paper, no. 1, washed. The mixtures of *n*-propanol: conc. aqueous ammonia (sp.gr. 0·880) used as solvents were (in vol.): no. 1, 90:10; no. 2, 80:20; no. 3, 70:30; no. 4, 60:40.)

		R_F value in solvent no.			
Monocarboxylic acids	x	1	2	3	4
$H.(CH_2)_x.COOH$	0	—	—	0·37	0·52
	1	0·13	—	0·37	0·52
	2	0·25	—	0·48	0·61
	3	0·33	—	0·57	0·69
	5	0·44	—	0·69	0·80
	7	0·52	—	0·73	0·84
	9	0·58	—	0·78	0·86
	13	0·62	—	0·82	0·88
Dicarboxylic acids					
$HOOC.(CH_2)_x.COOH$	1	—	0·04	0·09	0·23
	2	—	—	0·13	0·30
	3	—	0·07	0·16	0·34
	4	—	0·083	0·19	0·39
	6	0·02	0·18	0·29	0·49
	7	0·04	0·24	0·33	0·58
	8	0·07	0·28	0·44	0·65
Other acids					
Glycollic		—	—	0·26	0·39
Lactic (DL-)		—	—	0·395	0·48
Glyceric (DL-)		—	—	0·21	0·38
Maleic (*cis*)		—	—	0·08	0·21
Fumaric (*trans*)		—	—	0·11	0·23
Malic (DL-)		—	—	0·06	0·195
Tartaric (L-)		—	—	0·03	0·15
Tricarballylic		—	—	0·01	0·12
Aconitic (*trans*)		—	—	0·00	0·10
Citric		—	—	0·00	0·07

The results show that the amount of water in the solvent influences the R_F values in a regular manner; increasing amounts increase the R_F but the order in which the acids separate is unchanged. This is in agreement with the observations of Isherwood & Jermyn (1951) on the sugars. These authors showed that the plot of $\log_{10}\left(\frac{1}{R_F}-1\right)$ against $-\log_{10}(N)$, where N was the molar concentration of water in the solvent, was a straight line for each sugar. It is probable that a similar relationship exists for the ammonium salts of the organic acids but the data obtained in the present study were insufficient for a more detailed analysis.

Martin (1949) suggested the use of $RT \ln \alpha$ (where α is the partition coefficient) for comparing the chromatographic behaviour of members of a series of compounds, since the function might be expected to be the sum of contributions from all the constituent groups of a molecule, assuming that the effect of each of these on the distribution isotherm is independent of the others. Accordingly, in Fig. 2 values of $\log_{10}\left(\frac{1}{R_F}-1\right)$, which is proportional to the above function, have been plotted against the number of carbon atoms for the members of the two homologous series of organic acids for which data are available.

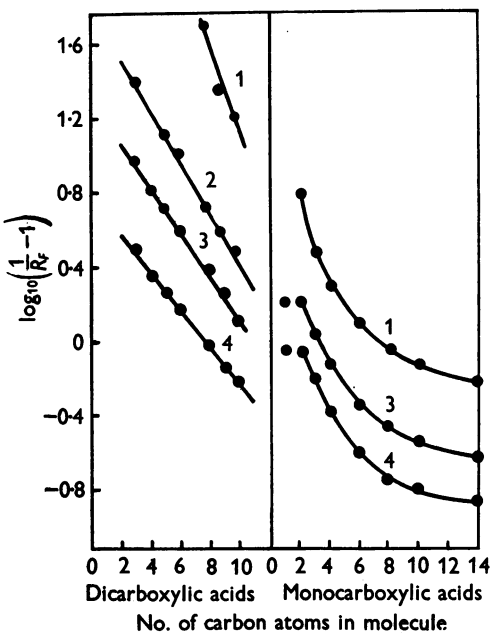

Fig. 2. Relationship between $\log_{10}\left(\frac{1}{R_F}-1\right)$ and the number of carbon atoms in the molecule for the monocarboxylic acids $CH_3.(CH_2)_n.COOH$, and for the dicarboxylic acids $HOOC.(CH_2)_n.COOH$. The mixtures of *n*-propanol: conc. NH_3 used as solvent were (by vol.): (1) 90:10; (2) 80:20; (3) 70:30; (4) 60:40.

It will be seen that the results for the dicarboxylic acids adhere closely to the relationship expected from the theory of Martin, the points for each solvent mixture falling closely upon a straight line. The results for the monocarboxylic acids, on the other hand, show a marked deviation from the expected linear relationship. For this series the value $\log_{10}\left(\frac{1}{R_F}-1\right)$ tends to approach a limiting value as the number of carbon atoms increases, an effect observed with all three solvents investigated. Other investigations showed that the form of this relationship was unaffected by the type of filter paper used (Whatman nos. 54, 541 and 1 unwashed were tried) and by the presence or absence of carbon dioxide in the chromatogram jar: there is no doubt that it is a characteristic of this homologous series of acids, the detailed interpretation of which is likely to prove of some theoretical interest. It should be noted that the decrease in hydrophilic character with increased number of methylene groups falls off to very small values in the region C_6–C_8; it may be significant that the ammonium salts of the acids begin to show the characteristic properties of soaps when this molecular size is reached. The unexpected chromatographic behaviour may thus reflect a marked change in the association of the molecules of the ammonium salts in the system (cf. Duin, 1953).

Quantitative analysis of ammonium salts of organic acids on paper chromatograms

The basis of the method is that if an acid is added to a neutralized solution of an acid indicator of which the anion is distinctively coloured, then the colour due to the anion is inversely proportional to the amount of acid added between 20 and 80 % neutralization. It is assumed that the acid is very much stronger than the indicator acid and that the pH range of the indicator in water does not lie above 10. Ammonium salts behave in the same way if a suitable indicator is used with a pH range above 10·0. This pH was determined by the highest concentration of free ammonia which might be expected (1×10^{-4} M) as a result of the reaction of the ammonium 10*n* on a paper chromatogram with the neutralized indicator. However, in practice the concentration of alkali needed to neutralize such indicators (1×10^{-3} M) made them too insensitive for the present purpose. A more convenient method was to use an indicator such as thymol blue (p*K* 9·6 in 35 % (v/v) aqueous ethanol) in conjunction with an aqueous-ethanolic medium. The effect of the ethanol was to depress the dissociation constant of the ammonium hydroxide liberated and at the same time raise the apparent dissociation constant of the indicator. The

combined effect was to decrease the effect of the liberated ammonia on the indicator to such an extent that it did not interfere seriously with estimation of the ammonium salts. Thymol blue was chosen as indicator because of its distinctive colour change from blue to yellow. In practice the indicator was combined with a colourless buffer (sodium glycinate) which had similar characteristics, the amount of indicator being set by the colorimeter used and the buffer by the strength of the ammonium salts to be measured. Glycine was chosen because its pK_A (9·8 in 35 % (v/v) aqueous ethanol) was very close to that of thymol blue (9·6 in the same solvent) and because it did not interact with any of the acids or with sugars. Boric acid, which was used in some of the early experiments, is known to give strongly acid complexes with hydroxy compounds which may be present in crude extracts from plant materials. The standard thymol blue-glycine reagent was designed for use with an EEL single photocell colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and for the estimation of quantities of acid from 20 to 120 μg. The results of adding sulphuric acid, ammonium sulphate and sodium hydroxide to the standard reagent are shown in Fig. 3. It is clear that the addition of ammonium sulphate has not the same effect as the addition of sulphuric acid but that the difference down to 20 % neutralization is small. Below 15 % neutralization the liberated ammonia influences the results. Between 20 and 80 % neutralization the curve is straight and this constitutes the working range of the method. Above 80 % the curve bends sharply because at the very alkaline pH a considerable excess of sodium hydroxide was needed to complete the neutralization. The small reading in acid solution was due to the yellow colour of the acid form of the indicator. The effect of adding ammonium sulphate in the presence of an excess of free ammonia is shown in Fig. 3 for reagents made up in 35 and 45 % (v/v) aqueous ethanol. The amount of ammonia added was equivalent to the maximum amount of ammonium sulphate which could be measured by the reagent (equivalent to 2·44 μl. of 0·5 N). It is clear that excess of ammonia noticeably affects both the reagent made up in 35 % aqueous ethanol and that made up in 45 % (v/v) aqueous ethanol but that the effect on the latter is much less marked. Free ammonia is not present in any quantity if the paper chromatograms are dried thoroughly and then the reagent made up in 35 % (v/v) aqueous ethanol is satisfactory. In addition, the linear portion of the curve is very much longer so that a wider range of concentrations can be measured. The quantitative results described later in this paper were all measured using this reagent.

The general effect on the subsequent paper chromatograms of washing the filter paper used has

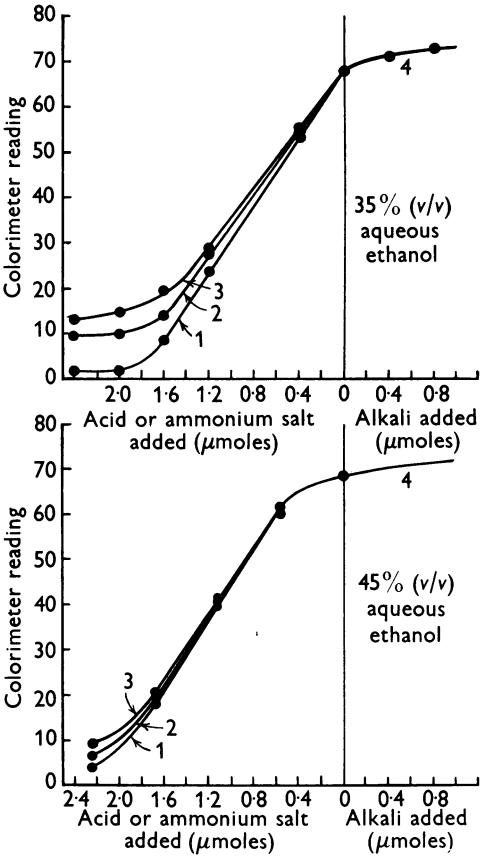

Fig. 3. The effect on colorimeter readings of adding (1) H_2SO_4, (2) $(NH_4)_2SO_4$, (3) an equimolar mixture of $(NH_4)_2SO_4$ and NH_3, (4) NaOH to the standard reagent in 35 % and 45 % (v/v) aqueous ethanol. For details see text.

already been briefly described in the Experimental section. In the case of the quantitative estimation of the acids, washed paper was essential. Early experiments with Whatman no. 1 which had not been washed or which had been washed with 2 N acetic acid and water gave very erratic results. Acids such as glyceric acid were impossible to estimate because there appeared to be a soluble compound in the paper which interfered with the reaction with the thymol blue reagent. Washing the paper with *n*-propanol:conc. aqueous ammonia (70:30, v/v) removed this inhibitor and then the ammonium salts could be readily estimated. Washing with a mixture of equal volumes of concentrated ammonia and water was equally effective and considerably cheaper.

The results for representative acids in different concentrations on washed Whatman no. 1 paper using purified *n*-propanol:conc. aqueous ammonia as solvent (70:30, v/v) are shown in Table 2.

Table 2. *Estimation of the ammonium salts of various acids on paper chromatograms (washed Whatman no. 1) using purified* n-*propanol:conc. aqueous ammonia (sp.gr. 0·880) as solvent (70:30, v/v) (single estimations)*

Acid	Amount added (μg.)	Amount found (μg.)	
Malic	16·6	16·6	
	33·2	34·8	
	66·4	70·0	
	83·0	81·0	
Succinic	14·6	13·8	—
	29·5	26·2	27·8
	44·2	44·0	48·0
	59·0	60·6	56·0
Acetic	24·2	22·4	
	48·4	45·6	
	72·6	71·5	

Table 3. *Analysis of mixtures of acetic, succinic and citric acids on washed Whatman no. 1 paper using* n-*propanol:conc. aqueous ammonia (sp.gr. 0·880) (70:30, v/v) as solvent (single estimations)*

Acetic	Succinic	Citric
Amount of acid added* (μg.)		
24·2	47·6	38·6
48·4	71·0	72·5
72·6	71·0	38·6
Amount of acid found (μg.)		
26·3	46·4	37·4
45·0	69·5	77·0
71·5	65·3	36·1

* The weights refer to free acids, although they were added as ammonium salts.

The amount of acid calculated from the colorimeter reading was not corrected for the losses incidental to the procedure, because to a very large extent the various errors balanced each other and the magnitude of the final correction would have been within the experimental error of reading the colorimeter. The loss due to the removal of the wet paper from the 3 ml. of water in the colorimeter tube before the addition of the thymol blue reagent causes a loss of about 3 % (approximately 0·1 ml. of liquid is removed from a total vol. of 3 ml.) in the total amount of acid present. The removal of this liquid causes the final volume after the addition of the thymol blue reagent to be smaller than if no paper had been added. This increases the colorimeter reading relative to a blank in which no paper had been introduced by 1–2 %. As mentioned previously, the blank readings must be made on paper which has been cut from a chromatogram freshly dried in the absence of carbon dioxide.

Some results on the analysis of made-up mixtures of acids are given in Table 3.

It was found in preliminary experiments that if too great a concentration of the mixture of ammonium salts was applied to the chromatogram, the 'overloading' caused serious errors in the estimation of the individual acids. Extraction of the individual acid spots and re-examination on a paper chromatogram showed that each contained a certain amount of the other acids. In addition, distortion of the shape of the spots which occurred made it difficult to distinguish closely related acids. In the present experiments the acids were added in no greater concentration than 0·3 N. In difficult cases where a small amount of one acid had to be determined in the presence of a large amount of another, it was found best to apply the mixture as a streak to the starting line of the chromatogram. By doing this a large volume of the solution could be examined without increasing the concentration above the safe level. The appropriate strip of paper after development of the chromatogram was extracted and the acid either determined directly on this extract or the concentrated extract re-examined on a paper chromatogram and estimated as described previously.

The recovery of acids added to extracts of cress seedlings (2 mg. of malic and 2 mg. of glyceric acids to 2 g. of cress seedlings) and separated from the bulk of the other solutes by the method of Isherwood (1946) and then estimated using the standard procedure was better than 95 %.

SUMMARY

1. Qualitative examination of a number of common aliphatic acids on a paper chromatogram using *n*-propanol:concentrated aqueous ammonia as solvent, has shown that many of them can be readily separated. In the present paper R_F values are given and the results are also expressed in terms of $\log_{10}\left(\frac{1}{R_F}-1\right)$ plotted against the number of carbon atoms in the molecule in order to throw light on relationships in the chromatographic behaviour of the acids.

2. A quantitative method is described for the estimation of the ammonium salts of the acids after they have been separated on a similar paper chromatogram. It is based on the effect of the ammonium salts on a thymol blue-glycine reagent which is insensitive to free ammonia. The change in colour of the thymol blue is inversely proportional to the concentration of the salt from about 80 % to about 20 % neutralization of the reagent. The accuracy of the method (single determinations) is about ± 10 % when 50 μg. of organic acid is present.

As regards one of us (F.A.I.) the work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Combination of some Vitamin B_{12}-like Compounds with Sow's Milk Whey and 'Intrinsic Factor' Concentrates

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In a previous communication from this laboratory, Gregory, Ford & Kon (1952) reported the presence in sow's milk of a substance that combined with vitamin B_{12} making it unavailable to assay micro-organisms. This 'binding factor' appeared to be associated with a particular fraction of whey proteins. Other naturally occurring materials have been shown to inactivate vitamin B_{12} in the same way. One of the first reports of this type of inactivation was that of Ternberg & Eakin (1949), who found that normal gastric juice and an aqueous extract of pig gastric mucosa contained a non-dialysable, heat-labile substance that combined with vitamin B_{12} and made it unavailable to assay micro-organisms. Prusoff, Meacham, Heinle & Welch (1950) fractionated extracts of desiccated pig stomach with ammonium sulphate and found that intrinsic factor activity was greatest in the fraction precipitated by 35–55 % saturation with ammonium sulphate. The ability of the various fractions to prevent the utilization of vitamin B_{12} by *Lactobacillus leichmannii* was investigated, but no figures were published in the communication.

The vitamin B_{12}-binding power of proteins such as egg albumin, globulins from blood and from soya beans, urease, lysozyme and an intrinsic factor concentrate has been investigated by Bird & Hoevet (1951). Only the intrinsic factor concentrate, however, showed any appreciable vitamin B_{12}-binding activity. Beerstecher & Altgelt (1951) observed a substance in saliva, similar to Castle's intrinsic factor from gastric juice in its ability to combine with and inactivate vitamin B_{12}. However, these two substances appear to differ in their heat stabilities (Beerstecher & Edmonds, 1951). Using dialysis techniques, in conjunction with microbiological assays, Rosenthal & Sarett (1952) have shown that vitamin B_{12} was present in a bound form in serum and that the serum was also capable of binding limited amounts of added vitamin B_{12}. Chow & Davis (1952) reported that yeast nucleic acid and heparin combined with vitamin B_{12}, but gastric juice was much more effective. Their measurements were made using radioactive vitamin B_{12} and no microbiological assays were carried out.

Compounds are known to exist, such as pseudovitamin B_{12}, first isolated by Pfiffner *et al.* (1951), and factors *A* and *B*, isolated from calf faeces by Ford & Porter (1952), that are not simple derivatives of vitamin B_{12}, but have 'vitamin B_{12} activity' for micro-organisms. Since the combination of vitamin B_{12} with intrinsic factor or other heat-labile substances is thought to be of importance in the metabolism of vitamin B_{12} (Ungley, 1951), it is of interest to find out whether the other vitamin B_{12}-like compounds also form a complex with intrinsic factor, particularly since pseudovitamin B_{12} has been shown to be clinically inactive in the treatment of pernicious anaemia (Pfiffner, Dion & Calkins, 1952). For this reason the microbiological inactivation of pseudovitamin B_{12} and of factors *A* and *B* by an 'intrinsic factor' concentrate and a concentrate from sow's milk has been investigated.

EXPERIMENTAL

Preparation of the sow's whey concentrate. Skimmed sow's milk was clotted with crystalline rennin at 40° and the curd removed. The whey was adjusted to pH 4·6 with glacial acetic acid and the proteins were fractionated by $(NH_4)_2SO_4$ saturation following the procedure used by Prusoff *et al.* (1950) for the concentration of intrinsic factor activity from powdered hog stomach. The proteins precipitated by